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14. ABSTRACT Thrombosis, or the abnormal clotting of the blood, is the leading cause of death in breast cancer patients. Thrombus formation is triggered factor VIIa from circulating blood encounters tissue factor (TF) on the surface of a cell, including many breast cancer cells and tumor-associated cells. It is important, therefore, to increase our understanding of the mechanisms by which TF activates the clotting cascade in breast cancer. The blood protein, antithrombin (AT), has long been thought to be the most important natural anticoagulant that targets steps of the clotting cascade downstream from TF:VIIa, but more recent work has suggested that AT can also inactivate factor VIIa bound to TF. Goals of this project included determining if AT can efficiently inhibit TF:VIIa complexes and if resulting factor VIIa-antithrombin (VIIa-AT) complexes can be detected in blood. The project was successful in determining that the TF:VIIa complex can efficiently be inactivated by AT, and furthermore was successful in quantifying VIIa-AT levels in blood.					
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SPECIAL EXPLANATORY NOTE:

This report is being prepared by Dr. James H. Morrissey instead of the PI (Francesca Antonaci). Ms. Antonaci was an MD/PhD graduate student at the University of Illinois at Urbana-Champaign (UIUC) during the tenure of this breast cancer fellowship. When she first received this fellowship she was working under the direction of Dr. David Shapiro at UIUC. She changed laboratories in 2003 to work with Dr. Morrissey, for which she obtained approval from the granting agency. Ms. Antonaci is currently on medical leave and is living outside the United States. Dr. Morrissey has prepared this final report and it represents, to the best of his knowledge, the work performed by Ms. Antonaci.

INTRODUCTION:

Thrombosis, or the abnormal clotting of the blood, is the leading cause of death in breast cancer patients, and 17.6% of breast cancer patients with metastases also have underlying thromboses. The step that triggers the formation of a thrombus occurs when factor VIIa from circulating blood encounters tissue factor (TF) on the surface of a cell, forming the TF:VIIa complex. Breast cancer cells and tumor-associated macrophages and endothelial cells express TF. Thrombus formation in these patients leads to such events as myocardial infarction, deep vein thrombosis, and stroke, pathological conditions which have high mortality rates; in fact, metastatic breast cancer cells may express as much as 1000 fold higher levels of TF than non-metastatic breast cancer cells, which has been shown to increase thrombogenic activity. The blood protein, antithrombin (AT), has long been thought to be the most important natural anticoagulant that targets steps of the clotting cascade downstream from TF:VIIa, but more recent work has suggested that AT can also inactivate factor VIIa bound to TF. Goals of this project included determining if AT can efficiently inhibit TF:VIIa complexes and if resulting factor VIIa-antithrombin (VIIa-AT) complexes can be detected in blood.

BODY:

Ms. Antonaci conducted studies on the kinetics of inhibition of TF:VIIa by AT in the presence of heparin. She obtained a second-order rate constant of inhibition of $2.5 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. Ms. Antonaci also discovered that the plasma protein, vitronectin, enhances AT inhibition of VIIa in the absence of TF. Normally, AT inhibits its target proteases by forming a highly stable covalent adduct with the proteases. Ms. Antonaci explored the ability of AT to reversibly inhibit VIIa in the absence of TF. She found that, in the presence of heparin, AT can inhibit VIIa alone by as much as 70%, and furthermore that full recovery of factor VIIa enzymatic activity can be recovered when the VIIa/AT mixtures were diluted. These rather preliminary findings suggest that VIIa may be susceptible to reversible inhibition by AT in the absence of TF, but irreversible inhibition by AT in the presence of TF.

Ms. Antonaci conducted rather extensive studies to quantify VIIa-AT levels in plasma. These studies provide baseline data on VIIa-AT levels in normal individuals, so in the future we can examine if VIIa-AT levels increased in breast cancer patients. She was able to quantify VIIa-AT levels in normal plasma using an ELISA method. VIIa-AT levels were found to be surprisingly high, constituting something like 1-3% of the total circulating factor VII antigen. This finding alone suggests that AT is a more important regulator of TF:VIIa activity in vivo than had previously been appreciated. She also used pull-down assays to visualize VIIa-AT complexes on western blots. She showed that the signal strength in these assays correlated with the VIIa-AT levels measured by ELISA. This work forms part of an abstract that is currently in press. We are currently preparing a full manuscript that will incorporate this portion of the work, and it will be submitted for publication soon.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstration that VIIa-AT complexes can be readily detected in human plasma and are present at surprisingly high concentrations
- Documentation of the rates of formation of VIIa-AT complexes when the TF:VIIa complex is reacted with AT in the presence of heparin
- Preliminary finding that, in the absence of TF, AT may be a reversible inhibitor of VIIa

REPORTABLE OUTCOMES:

Abstract in press, which includes some of Ms. Antonaci's work:

Smith SA, Antonaci FC, Woodhams BJ, and Morrissey JH. Factor VIIa-antithrombin complexes in human plasma. *J Thromb Haemost*, In Press.

Presentations:

The work described in the above abstract will be presented in a talk by Dr. S.A. Smith at the XX1st International Congress of the International Society on Thrombosis and Haemostasis in Geneva, Switzerland, on July 6-12, 2007.

Manuscripts:

A full manuscript is currently under preparation that encompasses the work described in the above abstract, for submission to a scientific journal.

CONCLUSION:

This work demonstrated that VIIa-AT complexes can be readily detected in human plasma and are present at surprisingly high concentrations. This finding suggests that AT is a more important regulator of TF:VIIa activity in vivo than had previously been appreciated. This work also provides a useful baseline for studies in which we can quantify VIIa-AT levels in breast cancer patients. The hypothesis that will be tested in such further studies is that high circulating levels of VIIa-AT complexes indirectly reveal high intravascular exposure of TF. We therefore expect high circulating levels of VIIa-AT complexes to be an indicator of risk of thrombosis in such cancer patients.

REFERENCE:

None.

APPENDICES:

Attached is a copy of the abstract mentioned above under REPORTABLE OUTCOMES.

Factor VIIa-Antithrombin Complexes in Human Plasma

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Introduction

Factor VIIa is susceptible to inhibition by antithrombin (AT) only when bound to tissue factor (TF:VIIa). VIIa-AT complexes lose affinity for TF, so we hypothesize that circulating VIIa-AT complexes reflect intravascular TF exposure. We measured VIIa-AT levels in plasma and their relationship to other markers of coagulation activation.

Methods

VIIa-AT levels were measured in citrated plasma samples from 123 healthy adults in a two-site ELISA using a capture monoclonal antibody (1172) that recognizes VIIa in complex with AT, and detected with polyclonal anti-AT antibodies. The ELISA did not recognize thrombin-AT or Xa-AT complexes. Plasma VIIa-AT complexes were also identified by immunoprecipitation with immobilized 1172 followed by SDS-PAGE/western blotting with anti-AT antibodies.

Results

Median VIIa-AT levels in normal plasma were 227 pM (range: 9.2 to 493), corresponding to about 2% of total plasma factor VII. VIIa-AT levels were significantly and positively correlated with plasma VIIa and VII antigen, with strongest correlation to VIIa (Pearson product moment 0.616, $p < 0.001$). VIIa-AT was statistically significantly correlated with CRP, AT and thrombin-antithrombin levels, although the relationships were less strong. VIIa-AT complexes were readily detectable by western blotting following immunoprecipitation from normal plasma, with signal strengths proportional to plasma VIIa-AT concentrations measured by ELISA.

Conclusions

VIIa-AT complexes are surprisingly abundant in plasma with a very wide normal range. The fact that they represent ~2% of plasma factor VII argues that AT is a more significant regulator of VIIa function and turnover in plasma than previously appreciated. VIIa-AT may also be an important indirect indicator of intravascular TF exposure.